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SOME FACTORS AFFECTING THE GROWTH AND DISTRIBUTION OF THE ALGAL ENDOSYMBIONTS OF *HYDRA VIRIDIS*

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Researches on the hydra-algae endosymbiosis over the last decade have produced a clearer understanding of the role of the symbiotic zoochlorellae. The works of Muscatine and Lenhoff (1965a, 1965b) have shown that green hydra survive and bud longer than aposymbiotic (algal-free) hydra under starvation conditions. Moreover, Muscatine (1965) and Muscatine and Lenhoff (1963) have demonstrated that the algae may release soluble, photosynthetically fixed material to the host's cells. Roffman and Lenhoff (1969) have shown that photosynthetically fixed $^{14}CO_2$ appears, with time, in all of the host's major biochemical fractions but predominates in the animal's glycogen pool. Analysis of the products released by the algae *in vitro* have shown them to consist mainly of the disaccharide, maltose (Muscatine, 1965). The assumption is that maltose is translocated to the host by the algae *in vivo* and serves as nutritional supplementation for the host during periods of starvation.

The ultrastructure of algae symbiotic with hydra has been studied by Park, Greenblatt, Mattern and Merril (1967) and Oshman (1967). These studies have shown that the algae are located in separate vacuoles within the digestive cells, and that the algae appear to be similar to free-living *Chlorella vulgaris,* a view advanced by Haffner (1925) and Beyernick (1890). Moreover the algae are seen to reside mainly in the base of the digestive cells (Whitney, 1907; Goetch, 1924; Haffner, 1925; Pardy and Muscatine, 1973).

Beyernick (1890) and Haffner (1925) claim to have cultured algae from green hydra, but repeated attempts by myself and others (Muscatine, personal communciation) to grow isolated hydra algae in pure culture have failed. As a result of the apparent inability to effect an axenic culture of the algal symbionts it is suggested that the symbiosis is obligate for the algae. Only recently has it been shown that the algae may derive nutrition from the host. Cook (1972) demonstrated that algae in hydra become radioactively labeled when the animals were fed ^{14}C labeled *Artemia* nauplii.

Current work has shown that aposymbiotic (algal-free) *Hydra viridis* are able to recognize potential algal endosymbionts and reject non-symbiotic algae (Pardy and Muscatine, 1973). These workers have shown that symbiotic algae injected into the gastrovascular cavity of aposymbiotic hydra are phagocytosed and transported to the base of the animals' digestive cells. With time the injected algae reestablish the symbiosis and restore the standing population of endosymbionts.

Virtually nothing is known as to how the endocellular algal flora may be regulated or modulated by the host. Unstudied is the effect of various environmental conditions on the size and distribution of the resident algal flora. This

paper describes the pattern of algal distribution in the hydra, how this pattern may be altered, and how continuous light, dark, and the animal's nutrition may affect the size of the algal population maintained by hydra.

Materials and Methods

Maintenance of hydra cultures

Stock cultures of *Hydra viridis* (Florida strain) were maintained in M solution (Muscatine and Lenhoff, 1965a) at room temperature and room illumination (approximately 50 foot-candles), hereafter referred to as ambient conditions. The animals were fed daily to repletion on freshly hatched *Artemia* nauplii. For dark experiments, animals were placed in 15 cm plastic petri dishes which were then wrapped with aluminum foil. These animals were exposed to no more than 2–4 minutes of light daily for counting, feeding and routine maintenance. In experiments requiring animals to be exposed to continuous illumination, animals in plastic petri dishes were placed on a white background 25 cm below two 20-watt Sylvania Gro-Lux lamps.

Technique for counting the number of algae in hydra

Endocellular algae were counted in digestive cells that had been isolated from hydra by maceration (David, 1973). In practice, either an individual or a piece of hydra was transferred to a drop of maceration fluid on a microscope slide. This solution consisted of glacial acetic acid, glycerine and water (1 : 1 : 13-v : v). After 5 minutes in this fluid the individual cells were teased apart with fine dissecting needles. Figure 1 is an example of a digestive cell prepared in this manner and demonstrates the presence of endocellular algae. The average number of algae/digestive cell was determined by counting directly the algae in digestive cells prepared by maceration.

In an exponentially growing population of hydra there are individuals in all states of development, of varying size, and possessing a variable number of budding hydranths. Thus the selection of a "standard" experimental animal is somewhat arbitrary. For the present work a standard hydra was defined as an animal that had two budding hydranths in an advanced state of growth.

To estimate the total number of algae in hydra, 10 standard animals were homogenized in 1 ml of M solution using a semi-micro tissue homogenizer. An aliquot of homogenate was transferred to a hemacytometer where the number of algae in the sample was determined. Counts from 5 groups of hydra were averaged and extrapolated to yield the average number of algae per hydra. In some experiments it was necessary to determine the standing crop of algae in an entire growing population of hydra. Since growing populations of hydra have individuals in various stages of development as mentioned earlier, a more useful index was found to be the number of algae per *hydranth*. Therefore the number of algae counted from homogenates of whole populations was expressed as the number of algae per hydranth.

Inspection of intact hydra reveals that the animals are not uniformally green. The central region of the hydra appear greener than the stalk and base. Such

an observation has been reported previously by Pardy and Muscatine (1973). As various environments and physiological factors might influence the distribution of algae within the animals, it was important to assess quantitatively the apparent heterogenous distribution of algal symbionts. To do this animals were divided into three zones (Pardy and Muscatine, 1973): Zone 1, hypostome and tentacles; Zone 2, central growing region; Zone 3, stalk and base. Zones were dissected from ten individual hydra (buds removed) and macerated separately. The number of algae in 20 digestive cells from each zone was counted. In experiments dealing with the effects of light and feeding on the number of algae per digestive cell (discussed below), only cells from Zone 2 were analyzed.

Effect of light, dark and feeding on the number of endocellular algae

To determine the effect of light and nutrition on the number of endocellular algae, cultures of hydra were maintained under the following conditions: (1) ambient light conditions—fed every 24 hours, (2) ambient light conditions—starved, (3) constant illumination—fed every 24 hours, (4) constant illumination—starved, (5) constant darkness—fed every 24 hours, (6) constant darkness—starved.

Animals for these experiments came initially from populations in exponential growth maintained under ambient light conditions and fed every 24 hours. Hydra from such cultures had 18 ± 3.1 (Zone 2) algae per digestive cell ($n = 100$). At 24-hour intervals 100 digestive cells (20 from each of 5 animals were selected from each of the experimental groups—Zone 2) were examined and the number of algae in each cell was recorded. The data were expressed as the mean number of algae $\pm$ s.d. per digestive cell.

Growth rate of hydra

Hydra's growth rate was measured according to the method of Loomis (1954). Standard animals with two buds were placed in 8 cm plastic petri dishes. Every morning the total number of hydranths was counted, the number recorded, and the animals fed to repletion. The average number of hydranths from duplicate experiments was plotted on semi-log paper against time. From a straight line fitted to these points, K, the growth rate constant was calculated using the standard equations for exponential growth.

Growth rate of endosymbiotic algae

Ten dishes each containing 20 hydranths were maintained in either continuous illumination or constant dark and fed every 24 hours. At 24-hour intervals following the start of the experiment for a total of 5 days, the total hydra population in a dish was harvested and homogenized in 1 ml of M solution. The number of algae in an aliquot of the homogenate was determined by using a hemacytometer as described earlier and the average number of algae from two dishes was plotted on semi-log paper against time. Growth rates of the algae were calculated in the same manner as those for hydra.

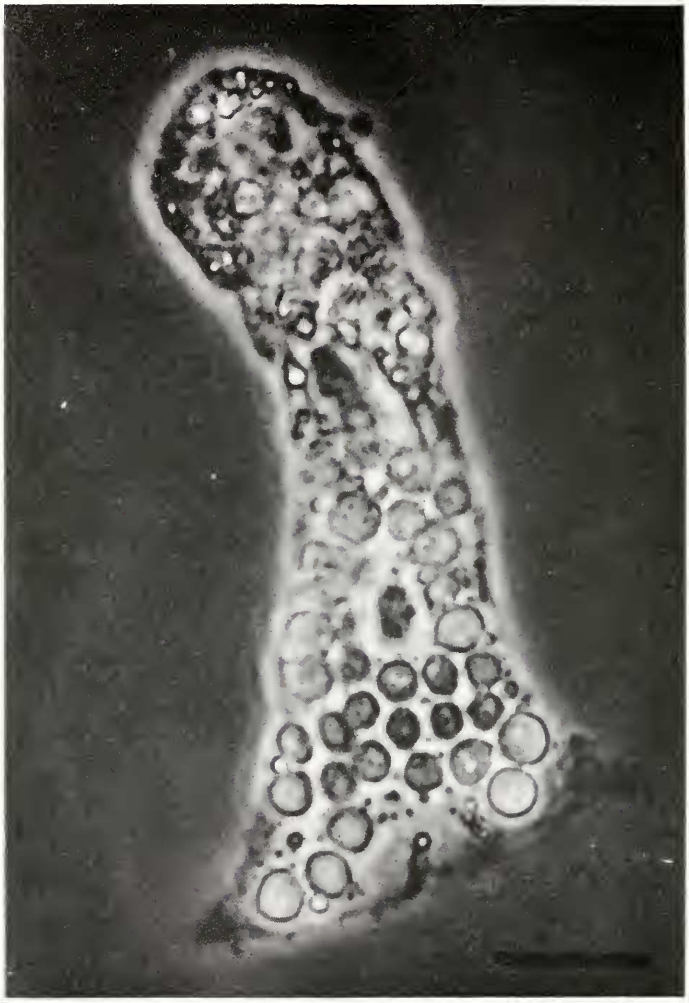

FIGURE 1. A digestive cell from green hydra prepared by maceration and photographed with phase optics. Symbiotic algae can be seen in the base of the cell. The animal cell nucleus with its prominent nucleolus is located centrally. Bar equals 10 microns.

RESULTS

Examination of cells from hydra prepared by maceration (Fig. 1) showed that the algal symbionts resided mainly in the base of the digestive cells. All digestive cells examined contained algae though individual digestive cells evidenced considerable range (2–30) in the number of algae contained in them. I found approximately $1.5 \times 10^5 \pm 3.7 \times 10^4$ algae per hydra in a standard intact *Hydra viridis,* a value closely agreeing with that of Pardy and Muscatine (1973).

Figure 2 is a histogram showing the distribution of algae in the three zones of hydra. It is evident that the algae are not uniformly distributed throughout the

animal. Zone 2, the central growing region, averaged approximately 19 algae per digestive cell, whereas Zones 1 and 3 had fewer algae cells, averaging about 12 symbionts per digestive cell.

Animals grown in continuous darkness became increasingly pale, however, I observed that Zone 3 appeared to be greener than Zones 1 and 2. As this condition was different from animals maintained in continuous light (see above), I

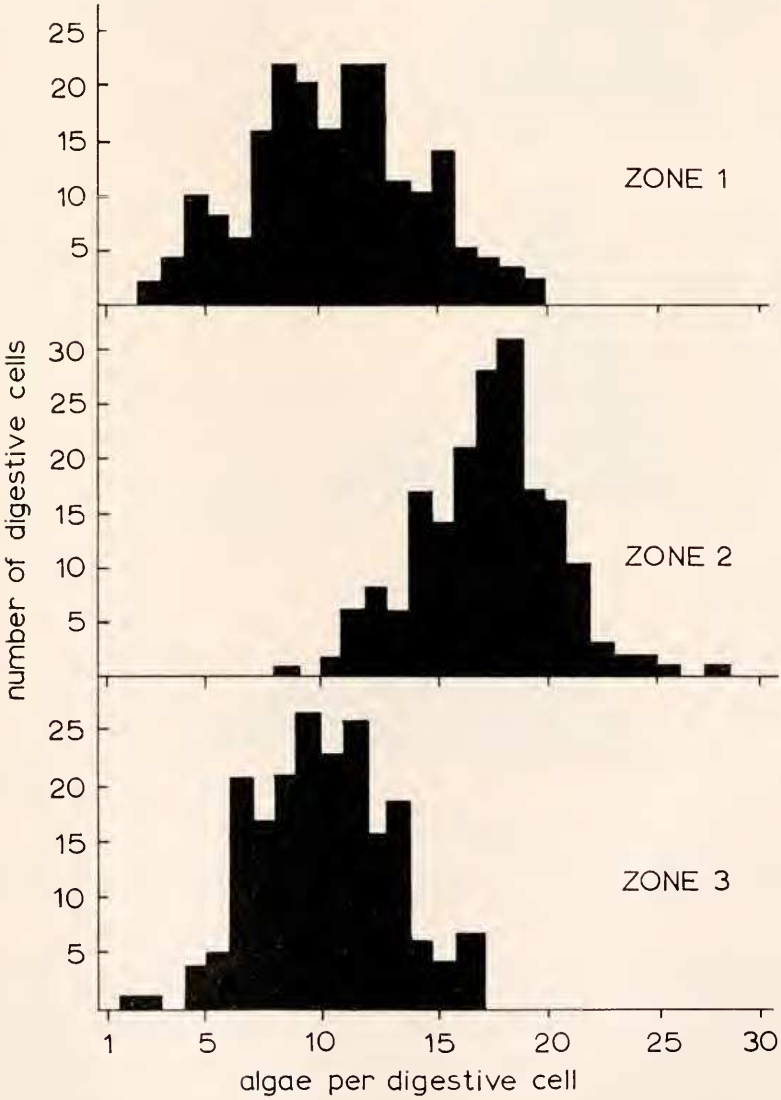

FIGURE 2. Histogram showing the distribution of algae in three zones of green hydra maintained in constant illumination and fed daily; Zone 1—tentacles and hypostome, Zone 2—central growth region, Zone 3—stalk and basal disc.

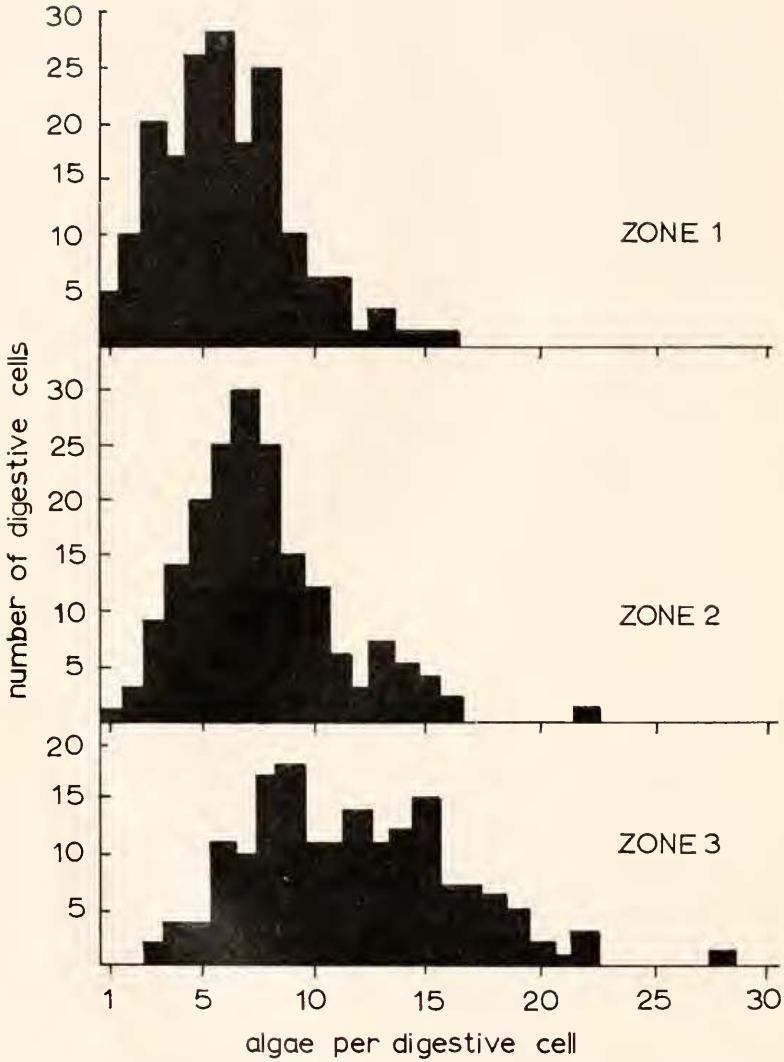

FIGURE 3. Histogram showing the distribution of algae in three zones of green hydra maintained in continuous dark for 6 days. Zones are the same as Figure 2.

examined the distribution of algae in the three zones from hydra grown in the dark. Figure 3 is a histogram of the distribution of algae from hydra grown in the absence of light for 16 days. Compared to the zones from hydra reared in continuous light (Fig. 2), the results shown in Figure 3 may be summarized as follows: Zones 1 and 2 have fewer algae (averaging approximately 6 and 7 per digestive cell, respectively). Zone 3 had approximately the same number of algae (11 symbionts per digestive cell) as hydra grown in continuous light. These data show that the numbers and proportions of algae in Zones 1 and 2 (Fig. 2)

are not fixed but may be altered by growing the animals in continuous darkness (Fig. 3). During growth in the dark, the number of algae are reduced in Zones 1 and 2, but apparently not in Zone 3.

Table I shows the effects of the various illumination and feeding conditions on the number of algae in digestive cells from hydra. Hydra which are fed and maintained under ambient conditions exhibited a nearly constant number of algae per digestive cell over the duration of the experiment. The remainder of the experimental results can be grouped in two classes: those in which there is a small, transient increase in algae followed by an approach to starting levels (Table I, ambient-starved, constant light-fed, constant light-starved), and those in which a pronounced, continuous decrease in the number of algae per digestive cell was observed (Table I, constant dark-fed, constant dark-starved).

TABLE I

Number of algae/digestive cell (Zone 2) from animals maintained under various conditions of light and feeding (see Materials and Methods). Data are expressed as mean ± s.d. of 100 digestive cells (20 from each of 5 hydra). Animals for the experiments were selected from a population of hydra that averaged 18 ± 3.1 algae/digestive cell (Zone 2) on day 0

Experimental condition	Day				
	1	2	3	4	5
Ambient-fed	18.2 ± 2.8	17.0 ± 2.0	17.0 ± 3.1	18.4 ± 2.6	17.9 ± 3.2
Ambient-starved	20.4 ± 3.1	20.2 ± 4.1	20.1 ± 3.6	18.3 ± 2.8	18.0 ± 3.0
Constant light-fed	18.1 ± 4.6	20.5 ± 4.7	19.6 ± 3.8	20.8 ± 4.1	19.1 ± 4.5
Constant light-starved	20.0 ± 4.1	21.0 ± 5.7	22.2 ± 3.8	21.8 ± 4.6	18.3 ± 3.3
Constant dark-fed	13.5 ± 3.8	12.3 ± 3.8	10.6 ± 3.7	6.8 ± 2.3	6.6 ± 3.1
Constant dark-starved	17.6 ± 3.8	17.0 ± 1.4	12.5 ± 3.4	11.0 ± 3.0	11.0 ± 3.2

Figure 4 is a semi-log plot of the increase in the number of algae and hydra in cultures maintained under continuous illumination and fed to repletion every 24 hours over a period of 4 days. The growth rate constant of hydra under these conditions was K = 0.358 and of the algae, K = 0.380. The doubling time for the hydra and algae was approximately 1.9 days.

The result of growing hydra under conditions of continuous dark while feeding every 24 hours is shown in Figure 5. It is evident that the hydra population keeps expanding exponentially though at a rate (K = 0.288) somewhat less than hydra maintained in continuous light (Fig. 2). The algae undergo only a very slight increase (K = 0.026) compared with those in the continuous light experiment (K = 0.380).

When the average number of algae per hydranth from populations of hydra maintained under continuous light and dark is plotted against time on semi-log coordinates, the curves shown in Figure 6 are obtained. From these graphs it is apparent that the average number of algae per hydranth remains nearly constant in populations grown under continuous illumination. Conversely, the number of algae per hydranth in hydra grown in continuous dark declines (K = − 0.232).

Inspection of Figures 5 and 6 suggests that the decrease of algae observed in hydra which are grown in the dark may be correlated with the exponential growth

of the animals. If the rate of algal decrease ($K = -0.232$) is corrected for the slight rate of algal division actually observed in the dark ($K = 0.026$) (Fig. 5), a new rate, $K = -0.258$ is obtained. This value represents the theoretical rate of algal decline to be expected in dark-grown hydra if the algae were to cease dividing. Hence the rate of algal decrease should equal the growth rate of the animal hosts. The theoretical rate of algal decreases given above ($K = 0.258$) approximates the rate of animal multiplication ($K = 0.288$) and indicates that the loss of algae from hydra reared in the dark probably results from dilution of the standing crop of algae brought about by repeated animal cell division.

The results from the experiment depicted in Table I (constant dark-fed), and Figures 4, 5 and 6 clearly emphasize the role of light in maintaining continuous maximum algal growth. Thus an experiment was devised to determine how long it would take the algae to repopulate hydra following depletion resulting from exponential-growth of the animals in the dark. Duplicate cultures of hydra were maintained in continuous darkness and fed every 24 hours. After 11 days it was determined that the hydra had approximately 7.1×10^3 algae per hydranth. The cultures were then placed in continuous light and the average number of algae per hydranth was determined every 24 hours for 4 days. Figure 7 shows that there

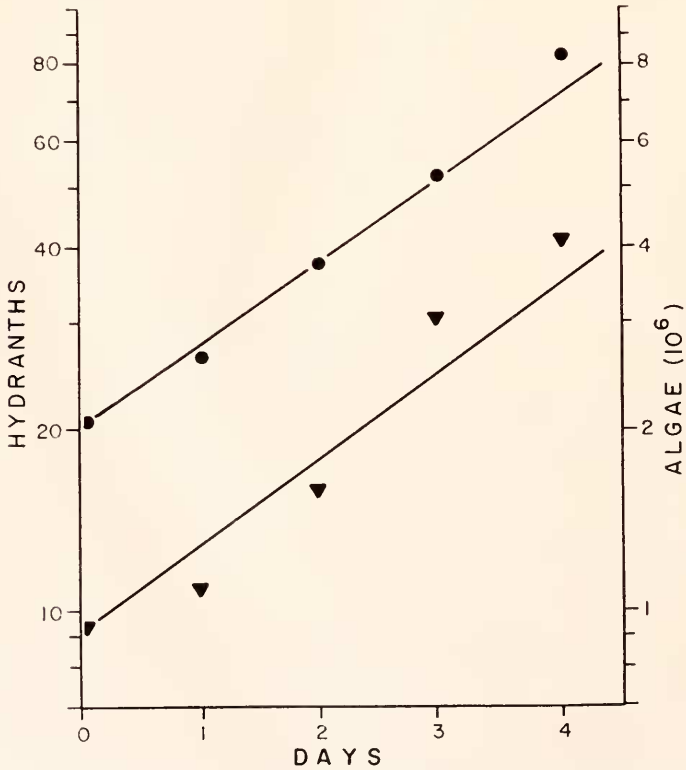

FIGURE 4. Growth of hydra and algae under continuous illumination and daily feeding—circles, hydranths; triangles, algae; growth rate of hydra, $K = 0.388$; growth rate of algae, $K = 0.380$.

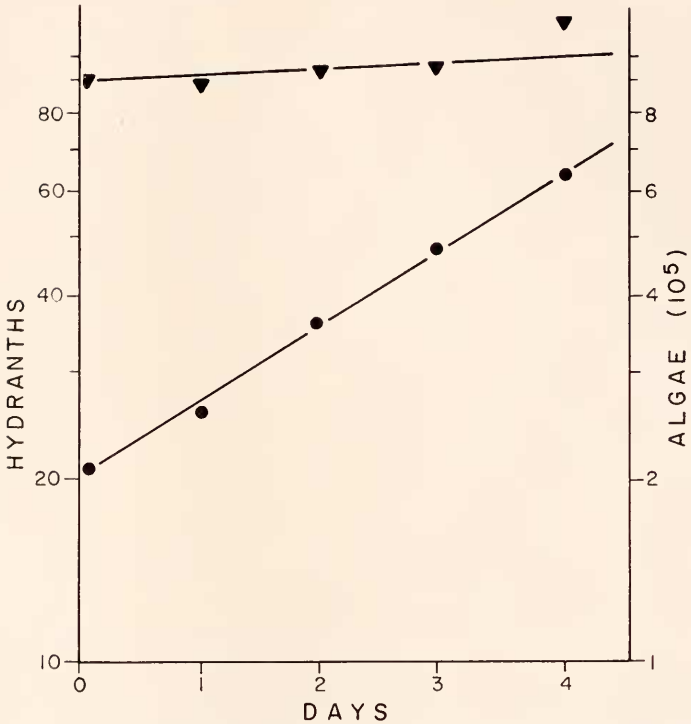

FIGURE 5. Growth of hydra and algae in continuous darkness and with daily feeding—circles, hydranths; triangles, algae; growth rate of hydra, K = 0.288; growth rate of algae, K = 0.026.

was a burst of algal multiplication over the first 2 days following the return of the animals to light. During this initial period the algae exhibited a growth rate of 1.37—over three times the typical algal rate. After the period of rapid multiplication the algal population reached and maintained a level typical of hydra grown in continuous light.

DISCUSSION

The effect of light and feeding on the growth of algae in hydra can be viewed as the consequence of two distinct but interrelated factors: the necessity of light for maximum algal multiplication, and tissue growth in hydra. Light has been shown to be a necessary condition for a variety of algal processes (photosynthesis, organic and inorganic nutrient assimilation, ion uptake) including division in certain strains of *Chlorella vulgaris* (Griffith, 1961). Algal multiplication in green hydra is strongly light dependent. Figure 5 shows that when hydra are transferred to the dark the algal population increased only slightly (K = 0.026). Under constant light and feeding, the number of hydra increases at an exponential rate of K = 0.358 and the algae increase at approximately the same rate (K = 0.380) (Fig. 4). These results are expected if animals in a growing population of hydra are to maintain a constant, optimum number of algae, and Table 1,

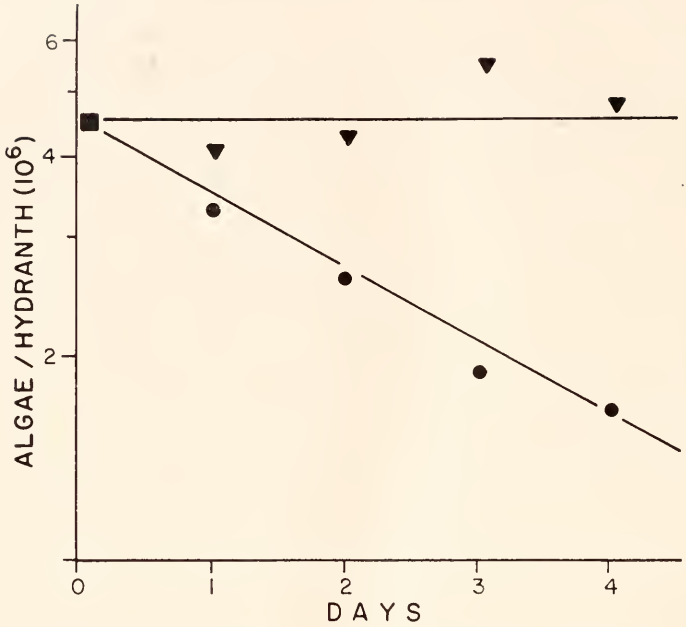

FIGURE 6. The number of algae per hydra in continuous light compared to the number of algae per hydra in animals maintained in continuous darkness. Both groups fed daily; the rate of decrease of algae, K = − 0.232; triangles, continuous light; circles, continuous dark.

ambient-fed constant light-fed, and Figure 4 indicate that the hydra do maintain a relatively constant number of algae during exponential growth in the light.

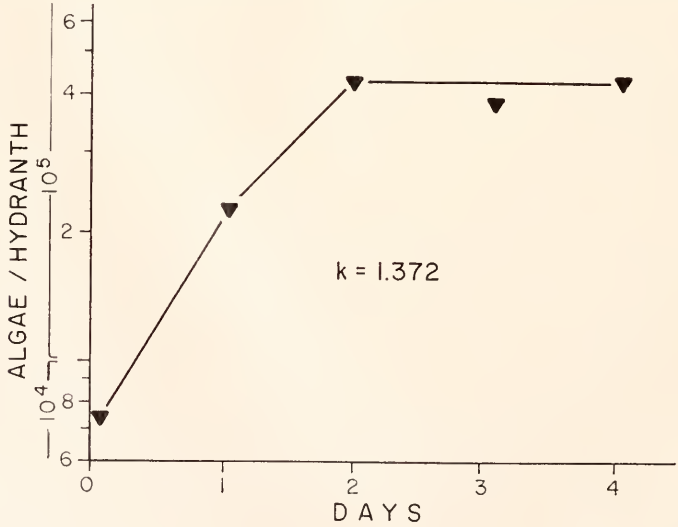

FIGURE 7. Growth of algae in hydra after being transferred to continuous light following 11 days in constant darkness; animals fed daily.

When hydra is reared in the absence of light, the number of algae per digestive cell (Zone 2) and per hydranth rapidly declines (Table I, constant dark-fed; Fig. 6). The algae continue to grow in the dark though very slowly (Fig. 5). The depopulation of algal symbionts observed in animals reared in the dark results from the growth and asexual reproduction of the host. The animals keep proliferating at a high rate relative to the algae and hence outgrow their symbionts.

Further support for this argument comes from Table I, constant dark-starved, which shows that when hydra are starved in the dark fewer algae are lost from the digestive cells than when hydra are fed (Table I, constant dark-fed). In starved hydra the rate of animal growth decreases (Muscatine, 1961) and with time the hydra cease multiplying altogether. In the absence of hydra growth in the dark, the apparent loss of algae also stops. I have kept starved animals in the dark for up to 10 days without observing any loss of green color.

It could be postulated that exponentially growing hydra maintained in the dark would eventually outgrow their algal symbionts. In fact, such an effect has not been observed. I have kept growing cultures of hydra in the dark for as long as four months with the animals maintaining a low level of infection that reproduces at the same rate as the hydra. It is possible that in the dark the algae assume a heterotrophic mode of nutrition and derive all of their energy by the assimilation of metabolites from the hydra. The total number of heterotrophic symbionts that are maintained under dark conditions would thus be determined by density-dependent factors such as competition for nutrition available from hydra. Cook (1972) has shown that transfer of material from hydra to algae takes place. Hydra kept in both light and dark were fed ^{14}C labeled *Artemia* nauplii. Assay of the algae after 48 hours showed that they had acquired 25–43% of the total radioactivity in the dark *versus* 22–26% in the light. While the nature of the translocated substance(s) is not known, Cook's work (1972) clearly demonstrates that a flow of material from hydra to the algae takes place. This material is probably the source of heterotrophic nutrition for the algae.

There is evidence suggesting that algae in the dark may be competing with hydra for metabolites. I have found that green hydra grows slower in the dark (Fig. 5, $K = 0.288$) than in the light (Fig. 4, $K = 0.358$) and that the animals growth rate may be depressed by as much as 20% in the dark. Thus in the dark there may be two levels of competition—between algae and between algae and host—the sum of which has the overall effect of depressing the growth of both symbiotic partners when they are in the dark. The absence of light, however, has an overall greater effect on the growth of the algae.

My data show that hydra reared in the light have most of their endosymbionts located in the central growing region (Fig. 2). This situation may reflect that some essential nutrient(s) supplied by the hydra cells of the growth region and needed for maximal algal growth are limiting in the hypostomal (Zone 1) and basal (Zone 3) regions. Alternatively, hydra may actively regulate algal mitosis via some hormone-like mechanism, promoting division in the growth region and/or suppressing it in the basal and hypostomal region. Finally, algal mitosis might in some way be influenced by digestive cell mitosis. While there is no evidence to support these ideas, the work of Campbell (1967) is suggestive. Campbell (1967) demonstrated that while mitosis occurs throughout the body of the animal,

the greatest amount of mitotic activity occurs in those cells (digestive, epithelial-muscular) of the central growing region with a substantially lower level in the base and hypostomal areas (Campbell, 1967). Recently, David and Campbell (1972) have found that the cell cycle of epithelial cells in the basal disk and peduncle of *Hydra attenuata* is longer than those in the growing regions. The zonal distribution of algae that I have found in green hydra appears to correspond to the pattern of actively mitosing cells found in other hydra. Possibly the processes which initiate or are involved in mitosis of the hydra cells promote algal division.

Hydra maintained in the dark exhibit a pattern of algal distribution (Fig. 2) differing from animals reared in the light (Fig. 3). In dark-reared animals there are more algae in the base (Zone 3) than in the other zones. Such a situation may be interpreted as follows: Loss of algae from hydra reared in the dark is a result of dilution of the standing crop of algae by the division of the digestive cells and is proportional to the growth rate of hydra. Because the animal cells constituting the various zones probably do not divide at the same rate (Campbell, 1967), the differential loss of algae observed in the various zones may reflect the differential mitotic activity of the digestive cells in these regions. The digestive cells of Zone 3 divide slower than the cells in the other zones and hence, in the dark, have proportionally more algae.

Though direct experimental evidence is lacking, it is generally agreed that the endosymbiotic algal population in hydra is under some kind of regulation. The algae do not over-grow their hosts and seem to maintain a fairly constant number providing there is adequate light (Table I, ambient-fed, ambient-starved, constant light-fed). Possibly hydra actively regulates the division of its algal flora, maintaining a population compatible with the physiology and metabolism of the digestive cells. Alternatively, the ultimate number of algae in digestive cells (in the presence of light) may be density dependent. The quantity of algae could be limited either by competition among fellow symbionts for growth-promoting substances supplied by the hydra or by growth-inhibiting factors released by the algae themselves. Hydra transferred to constant light, whether fed or starved, show a transient increase in the number of algae per digestive cell (Table I, constant light-fed, constant light-starved). Animals starved under ambient conditions also show this effect. If the algae are regulated by hydra, the slight algal increase observed may represent a temporary disruption in the control of algal division by the host or by a response to a change in physiological conditions, *e.g.*, feeding and light.

Hydra depleted of their algae as a result of rearing them in the dark experience a rapid repopulation by their symbionts when the animals are returned to the light (Fig. 7). Under these conditions the algae multiply at a rate nearly four times greater than under normal conditions. Rapid growth of the algae continues until the population reaches normal levels. Fast growth followed by plateauing under the conditions just discussed could be interpreted as either a result of density-dependent phenomena, active regulation by the hydra, or a combination of these processes. That light apparently triggers fast growth following prolonged maintenance in the dark is consistant with the behavior of other *Chlorella*. Griffith (1961) reported that cells of *Chlorella vulgaris* maintained in the dark accumulate

in a predivision phase. Such cells undergo rapid, multiple division when placed in the light.

I would like to dedicate this work to the memory of Dr. Berton Roffman, whose promising career was ended by his tragic and untimely death in 1972. Dr. Roffman had just begun an exhaustive biochemical study of the hydra-algae symbioses.

Summary

1. Under conditions of continuous illumination and daily feeding, individual specimens of *Hydra viridis* possess approximately 1.5×10^5 endosymbiotic algae.

2. The endosymbiotic algae are found in greatest abundance in those digestive cells constituting the central growth region (Zone 2) of the hydra (≈ 19 algae/digestive cell) with fewer algal cells (≈ 12 algae/digestive cell) residing in the hypostome, tentacles (Zone 1) and stalk and basal disc (Zone 3).

3. Under steady-state growth in the light, the algae reproduce at approximately the same rate as the animal hosts—K = 0.380 and 0.385, respectively.

4. Endosymbiotic algae require light for maximum reproduction and green hydra grown in the dark show a decrease in the number of their algal symbionts. The rate of algal decline in the dark is believed to result from a dilution of the standing crop by the continuous growth of the animal tissues. Support for this theory is based on the observation that the rate of algal decline in the dark (K = 0.258) approximates the rate of animal growth (K = 0.288).

5. The algae in hydra maintained in the dark exhibit a different pattern of distribution in the host than those in the light. Apparently loss of symbionts in the dark takes place differentially with proportionately more algae being lost from the tentacles, hypostome and growth region than from the stalk and basal disc.

6. When hydra that have been maintained in the dark are returned to the light, their endosymbiotic algae undergo a rapid multiplication and repopulate the host in approximately two days.

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